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REVIEW

Therapeutic Strategies in Alzheimer's Disease: M1 Muscarinic Agonists

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ABSTRACT—The cholinergic hypofunction in Alzheimer's disease (AD) appears to be linked with two other major hallmarks of this disease, β-amyloid and hyperphosphorylated *tau* protein. Formation of β-amyloids might impair the coupling of M1 muscarinic acetylcholine receptors (mAChR) with G-proteins. This can lead to decreased signal transduction, a decrease of trophic and non-amyloidogenic amyloid precursor protein (APPs) and generation of more β-amyloids, aggravating further the cholinergic deficiency. This review is an attempt to explore the M1 mAChR regulation of β-amyloid metabolism, *tau* hyperphosphorylation and cognitive functions. The therapeutic potential of M1-selective muscarinic agonists including AF102B, AF150(S), AF267B (the AF series) is evaluated and compared, when possible, with several FDA-approved acetylcholinesterase inhibitors. These M1 agonists can elevate APPs, decrease *tau* protein phosphorylation/hyperphosphorylation in vitro and in vivo and restore cognitive impairments in several animal models for AD. Except for the M1 agonists, no other compounds were reported yet with combined effects; e.g., amelioration of cognition dysfunction and beneficial modulation of APPs/β-amyloid together with *tau* hyperphosphorylation/phosphorylation. This property of M1 agonists to alter different aspects associated with AD pathogenesis could represent the most remarkable clinical value of such drugs.

Keywords: Alzheimer's disease, M1 muscarinic agonist, Animal model, β-Amyloid, *Tau*

1. Introduction

Neurofibrillary tangles, amyloid plaques containing the β-amyloid peptide (Aβ), and degeneration of cholinergic neurons that ascend from the basal forebrain to cortical and hippocampal areas are three major hallmarks in Alzheimer's disease (AD), the major cause of dementia in the elderly (reviewed in refs. 1 – 3).

The hypothesis that cholinergic hypofunction contributes to cognitive deficits in patients with AD has prompted the design of novel treatment strategies designed to restore lost cholinergic function. Two major strategies were extensively exploited so far via inhibition of acetylcholine (ACh) hydrolysis by acetylcholinesterase inhibitors (AChE-Is) and activation of muscarinic receptors (mAChR) by muscarinic agonists, respectively. The FDA has already approved 3 AChE-Is for treatment of cognitive impairments in AD and several more AChE-Is are at various stages of development or approval. Among the 5 subtypes of mAChR known so far, the M1 mAChR, predominant in the cerebral cortex and hippocampus, has attracted significant interest due to its major role in cognitive processing relevant to AD, in particular short-term memory (1 – 4). Notably, M1 mAChR is relatively unchanged in AD (3 – 7, but *vide infra*) and therefore may serve as a target for rational drug design of cognitive enhancers (e.g., M1 muscarinic agonists). M1 muscarinic agonists may offer an advantage in treating AD because they might allow the continuation of symptomatic treatment of cognitive decline when AChE-Is may no longer work. This is based on the assumption that M1 muscarinic agonists that activate postsynaptic M1 mAChR do not require the production and release of ACh from presynaptic terminals. Thus this strategy should be less limited, in principle, by the extent of degeneration of presynaptic cholinergic projections to the frontal cortex and hippocampus as observed in a progressive disease such as AD (6 – 9). However, some muscarinic agonists have major clinical limitations, lack M1 selectivity and showed disappointing clinical results in AD (8, *vide infra*). Therefore, the proof of the clinical concept utilizing the muscarinic approach could not be tested

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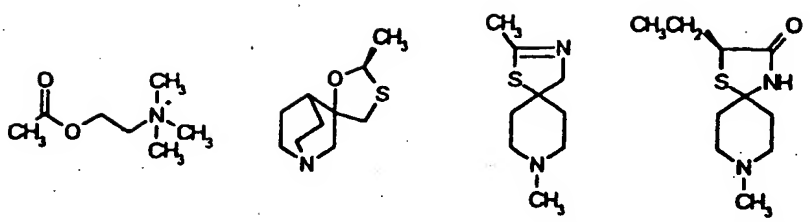

Fig. 1. The AF series vs acetylcholine.

appropriately.

Several centrally active muscarinic agonists were reported. These compounds can be classified as: a) M1 functionally selective including: AF102B (Cevimeline, the first reported M1 agonist); AF150(S) (reviewed in refs. 4, 6, 8) and AF267B (Fig. 1), talsaclidine (10), YM796 (11), CI-1017 (12); b) Higher selectivity for M4 versus M1 mAChR: xanomeline (13, 14); c) Non-selective: milameline (15); d) Partial agonists with limited selectivity for M1 mAChR: sabcomeline (SB-202026) (16, 17) and alvameline (LU 25-109), a very weak partial M1 agonist with M3 antagonist profile (18).

While ACh is a highly flexible molecule capable of attaining numerous conformations with the same energy, the AF series (Fig. 1), invented and studied by our group, are rigid analogs of this neurotransmitter. This rigidity may in principle limit the interaction of such compounds with the 5 mAChR subtypes, resulting in ligands selective for one or more of these subtypes. Based on computer modeling and docking studies, a hypothetical M1 muscarinic pharmacophore can be defined (6). Notably, this pharmacophore is preserved in the highly rigid compounds shown in Fig. 1 (6). Theoretically the rigid structure of the ligand may limit the conformational freedom in the ligand-receptor complex, which, in turn, may alter the affinity of M1 mAChR to form the ternary complex agonist-receptor-G-protein. In this context, we have shown that the functional selectivity of these agonists towards the M1 mAChR is preserved both at the level of the M1 mAChR as well as along select signal transduction pathways. This can be detected through activation of only distinct sets of G-proteins (Gq/11 but not Gs), and signal transduction pathways mediated by M1 mAChR. Such a ligand-induced select signaling might be clinically beneficial due to a relevant altered signal transduction in AD (6, 19 and *vide infra*).

Recent evidence indicates that M1 agonists could be useful not only in a symptomatic treatment but may also provide limited causal therapy in AD. Thus a relation between the formation of $A\beta$ peptide and amyloid plaques, *tau* phosphorylation/hyperphosphorylation and the loss of cholinergic function in AD brains was reported (reviewed in refs. 2, 4, 7, 8, 20–23). The text below is an attempt to explore these findings and the therapeutic potential of M1 agonists utilizing data accumulated on such compounds.

2. M1 agonists, $A\beta$ processing and prevention of apoptosis induced by $A\beta$

At least two major pathways control the processing of amyloid precursor proteins (APP): a) cleavage by an unknown secretase termed "α-secretase" of APP in the middle of its β-amyloid region to produce the secreted, neurotrophic and neuroprotective APP fragment (α-APPs); b) cleavage to form the $A\beta$ peptide, a major component of the amyloid plaques, via activation of β- and γ-secretases (reviewed in ref. 20; see also Fig. 2).

Stimulation of M1 mAChRs can increase formation (α-APPs), preventing the formation of $A\beta$ peptide. M1 agonists may prevent $A\beta$ formation by selectively promoting the "α-secretase" processing pathway in AD (Fig. 2). In this context, an increased secretion of α-APPs in various in vitro systems resulted in decreased synthesis of $A\beta$ following treatment with muscarinic agonists (28, 29). Several studies confirmed that α-APPs secretion in vitro is enhanced following activation of M1 mAChR with muscarinic agonists (26, 27, 30–34) (Table 1). Remarkably, M1-selective agonists may alter APP processing in the cortex and hippocampus where M1 mAChRs are abundant (33, 34).

Muscarinic stimulation activates at least two transduction pathways that lead to α-APPs secretion, protein kinase C (PKC)-dependent and mitogen-activated protein kinase-dependent (MAPK) pathways (Fig. 2A). These pathways operate in parallel and converge with transduction pathways of neurotrophins, resulting in enhancement of APPs secretion when both muscarinic agonist and neurotrophins stimulate their respective receptors (35).

Studies in vivo support the relation between the cholinergic system and $A\beta$ metabolism (Table 1). Such findings strengthen in vitro reports about a tight linkage between

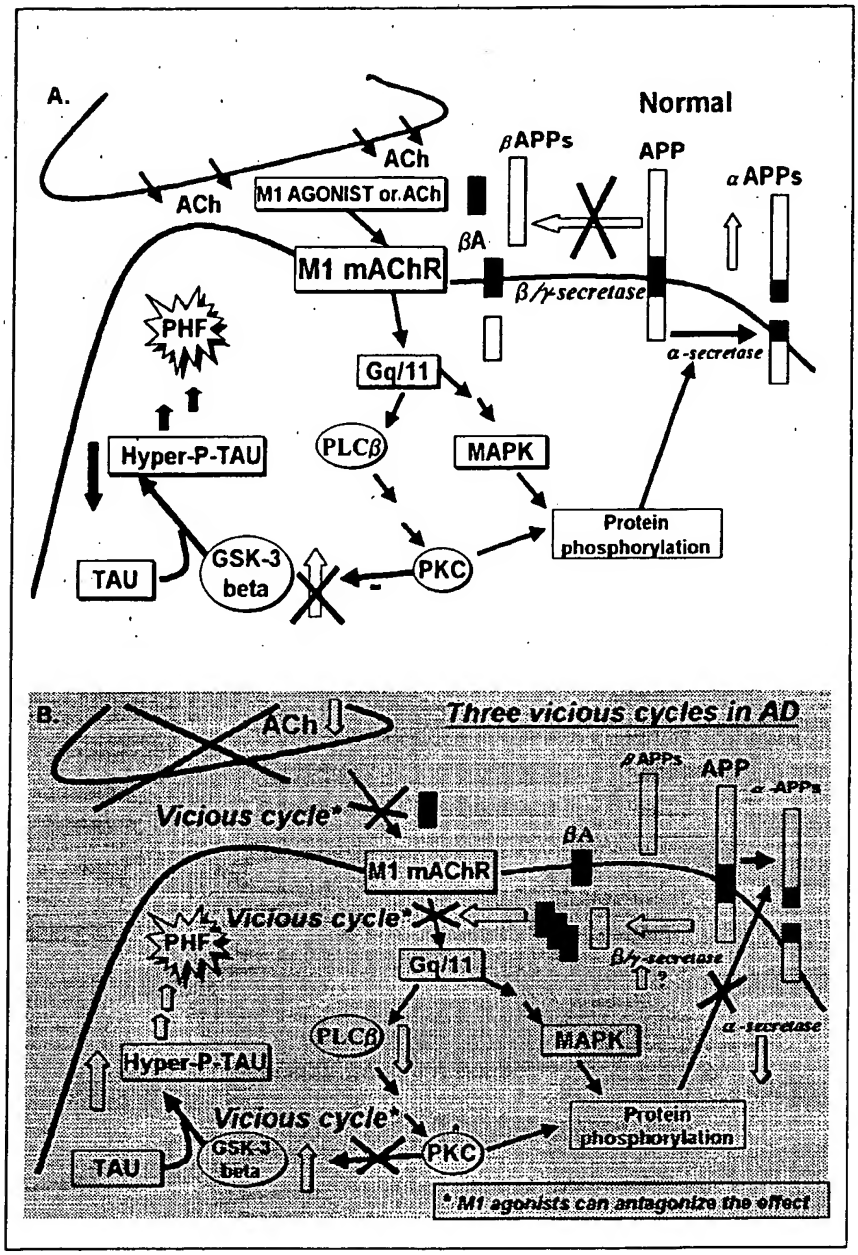

Fig. 2. The linkage of M1 mAChR, β-amyloid and *tau* phosphorylation. A: In a normal synapse, M1 agonists or ACh (released presynaptically) bind to M1 mAChR and form the complex agonist-receptor-Gq/11. α-APPs are increased following this interaction (24–28) via PLC-β-dependent and MAPK-dependent pathways (35). β-Amyloid levels are reduced following activation of M1 mAChR (28, 29). *Tau* protein hyperphosphorylation is also decreased by activating PKC that inhibits the constitutively activated GSK-3β (and/or inhibiting other kinases or upregulating phosphatases) (44–48). B: Three vicious cyles that link the cholinergic hypofunction with β-amyloid and *tau* phosphorylation. A cholinergic hypofunction in AD may lead to formation of β-amyloid which might impair the coupling of M1 mAChR with G-proteins (2, 67). This disruption in coupling can lead to decreased signal transduction, to a reduction in levels of trophic amyloid precursor proteins (α-APPs) and generation of more β-amyloid that can also suppress ACh synthesis and release (68, 69) aggravating further the cholinergic deficiency (2, 8, 65, 67). These "vicious cycles", a presynaptic and a postsynaptic one, may be inhibited, in principle, by M1-selective agonists. PKC, protein kinase C; HYPER-P-TAU, hyperphosphorylated *tau* proteins; PLC, phospholipase C; PHF, paired helical filaments.

Table 1. Cholinergic effects and APP/β-amyloid processing

Study (Ref.)	α-APPs	β-Amyloids
Muscarinic agonists (in vitro) (24–35)	Increase (24–35)	Decrease (28, 29)
Swedish Familiar AD (APP670/671 mutation) (80)	CSF: α-APPs is reduced and correlates with cognitive impairment.	Aβ (1–42) is elevated.
Tg mice (Swedish Familiar AD) +/− PKC activation (41)	PKC-activation leads to: α-APPs – No change β-APPs – reduction	Aβ (1–42) is elevated. PKC-activation leads to Aβ (1–42) reduction.
AF64A-treated rats (i.c.v.) (36)	H: α-APPs is reduced. APP-M is elevated.	Not tested
192 IgG-saporin lesioned-rats (37)	CT: α-APPs is reduced. APP-M is elevated.	Not reported
192 IgG-saporin lesioned-rabbits (39)	Not reported	CT: Aβ (1–42) is 8-fold elevated. Aβ (1–40) is 2.5-fold elevated.
192 IgG-saporin lesioned-rats; +/− RS-86 (limited electivity for M1 mAChR) (38)	−RS86 CT, H: APP-M is elevated. CSF: α-APPs is decreased. +RS86 CT, H: APP-M is decreased. CSF: α-APPs is elevated.	Not reported Elevated APP-M correlates with cognitive impairment.
AD patients (77–79)		AF102B (77) and tetrahydroaminoacridine (78) decrease significantly CSF $A\beta_{total}$. Physostigmine and hydroxychloroquine were inactive (77). Donepezil and galanthamine were inactive (79).

APP-M = membrane-bound APP, CT = Cortex, H = Hippocampus.

APP processing and ACh. Thus a chronic cholinergic hypofunction in rats induced by either the cholinotoxin AF64A (36) or the immunotoxin 192 IgG-saporin produced a decrease in α-APPs that may indicate a reduction in α-secretase activity (37, 38). Furthermore, RS-86 (a muscarinic agonist with limited M1 selectivity) attenuated the 192 IgG-saporin effects on α-APPs (38). In rabbits, where the sequence of Aβ (1–42) is similar to humans, a chronic cholinergic hypofunction elevated Aβ in the cortex and hippocampus (39). Additionally, activation of PKC reduced Aβ in transgenic (Tg) mice that produce elevated human Aβ (1–42) (40). In this context, several laboratories are evaluating the effects of M1 muscarinic agonists in such Tg mice. The hypothesis in such studies is that a reduced Aβ should occur in such animals, following stimulation of M1 mAChR since PKC activation is mediated also by this receptor (Fig. 2A).

Another aspect that appears relevant for the therapeutic potential of M1 agonists is their effects on apoptotic cell death induced by various insults such as growth factors deprivation alone or in combination with Aβ peptides (40). In this context, starvation of PC12M1 cells (PC12 cells stably transfected with M1 mAChR) decreases cell viability by 40%, and further addition of Aβ (25–35) (1–25 μM) reduces cell viability by 60%. AF150(S), AF267B and carbachol inhibited death of both starved and Aβ-treated cells. A 200% increase in apoptotic cells was detected after starvation of PC12 and PC12M1 cells, with a further increase of 50% after treatment with Aβ (25–35) or Aβ (1–42). In PC12M1, but not PC12 cells, these muscarinic agonists completely reversed both starvation- and Aβ-induced apoptosis, indicating activation of M1 mAChR. In addition, after starvation or Aβ peptides, the fraction of apoptotic cell population was decreased by muscarinic agonists and this effect was blocked by atropine indicating the involvement of mAChR. Additionally, following starvation or Aβ addition to PC12M1 cells, the transcription factor p53 was translocated from the cytoplasm to the nucleus in a time-dependent manner. Increased levels of immunoprecipitated p53 were observed following Aβ addition with a maximum at 4–5 h, and AF150(S) attenuated this effect. In summary, M1 agonists protect PC12M1 cells from apoptosis induced either by starvation alone or combined with Aβ peptides (40).

3. M1 mAChR-dephosphorylation of *tau* proteins

Tau microtubule-associated protein is neuronal specific,

and its expression is necessary for neurites outgrowth (42, 43). Hyperphosphorylated *tau* proteins are the principal fibrous component of the neurofibrillary tangle pathology in AD (reviewed in ref. 43). Activation of M1 mAChRs decreases *tau* protein phosphorylation. This was first shown in PC12M1 cells (44) and confirmed in vitro (cell cultures) (45, 46) and in vivo [in apolipoprotein E (ApoE)-deficient mice] (47). ApoE deficiency results in hyperphosphorylation of a distinct *tau* domain whose excess phosphorylation can be reduced by M1 muscarinic treatment (47). It can be deduced that activation of M1 mAChRs might provide a novel treatment strategy for AD by modifying *tau* processing in the brain (47). The effects on *tau* proteins suggest a link between M1 mAChR-mediated signal transduction system(s), and the neuronal cytoskeleton via regulation of phosphorylation of *tau* microtubule-associated protein (44). Moreover, this may indicate a dual role for M1 agonists: as inhibitors of a "vicious cycles" induced by Aβ and over-activation of certain kinases (e.g., GSK3-β) (Fig. 2B) and/or down-regulation of phosphatases, respectively (8, 43 – 48).

4. The evaluation of M1 muscarinic agonists in animal models for AD

Due to the unknown etiology of AD, no homologous animal model has been developed so far. Under such limitations, a multitude of animal models, mimicking different aspects of this disease, are needed to increase the predictive value of a potential drug to be used in AD. Several M1 agonists, including the AF series, were tested in various animal models (reviewed in refs. 4, 6 and 8). In this context, the M1 agonists from the AF series restored memory and learning deficits in several animal models that mimic cholinergic and/or other deficits reported in AD, without producing adverse central and peripheral side effects at effective doses and showing a relatively wide safety margin (>200 – 500 fold). The extensive preclinical database accumulated indicates that these compounds have fewer adverse effects and a higher safety margin in animal studies compared with other compounds of the same class (reviewed in refs. 4, 6 and 8). For illustrative purposes the effects of AF150(S) in ApoE-deficient mice and in the aged *Microcebus murinus* are described in more detail below.

4.1. Studies in ApoE-deficient mice: AF150(S) and rivastigmine

ApoE-deficient mice have memory deficits, synaptic loss of basal forebrain cholinergic projections and hyper-phosphorylation of distinct epitopes of the microtubule-associated protein *tau*. These impairments are restored by a prolonged treatment with AF150(S) (47, 49). AF150(S) completely abolished working memory impairments in this model in a Morris Water Maze test (49). Furthermore, this cognitive improvement in ApoE-deficient mice was associated with a parallel restoration to control values of reduced choline acetyltransferase and AChE and elevated M1 mAChR brain levels, respectively (49). These findings indicate that M1 agonists may have neurotrophic effects in vivo. Notably, under similar experimental conditions rivastigmine restored only cognitive impairments, but not AChE levels (50). From this and the study below, it can be anticipated that no tolerance is expected to occur in AD patients treated with partial M1 agonists such as AF150(S).

4.2. The effects of AF150(S) in the aged primate *Microcebus murinus*

The aged primate *Microcebus murinus* in captivity develops parenchymal amyloid plaques and abnormally phosphorylated *tau* proteins within the cortex (51 – 53). Neuronal loss, a basal cholinergic degeneration and specific behavioural and cognitive impairments accompany these pathological features. This natural animal model mimics in fact most of the neuropathology of AD (51 – 53). In this model a long-term chronic treatment with AF150(S) remarkably improved the behavior and cognition of the animals vs their baseline and also vs aged-matched microcebes. Notably, this is the first study that evaluated chronic drug treatment on cognition and behavior for a period of 18 months including a "drug holiday paradigm" in this unique model. The effects of AF150(S) do not diminish with time; on the contrary, a progressive increase in performance and in the number of responders is observed. This may indicate that AF150(S) might have a disease-modifying property in this animal model. It remains to be seen whether such potential property can be detected also in the brain pathology of the treated animals vs the control vehicle-treated. This supports AF150(S) as a candidate drug in AD that is not producing tolerance following such long term chronic treatment and the aged *Microcebus murinus* as a highly relevant model of the disease (54).

5. Why did some muscarinic agonists show disappointing results in AD?

Given the plethora of beneficial effects described above, a major question one can ask is why some of the tested muscarinic agonists failed in clinical trials in AD patients. Is this a unique case where either the side effects were too severe, the therapeutic potential too weak and/or the therapeutic concept was wrong?

The text below is an attempt to provide some sensible answers to this question by utilizing drugs that showed some positive effects in AD patients. In this context, AChE-Is (tacrine, donepezil and rivastigmine) are the only approved FDA drugs that show beneficial, albeit limited,

symptomatic effects on cognitive dysfunctions in AD patients (55).

It is not reasonable to presume that when compared with M1 agonists, only the AChE-Is should be effective in AD since: a) in general, the effect of the AChE-Is is mediated via increased synaptic concentration of ACh, and that by activation of M1 mAChR enhances memory and learning processing; b) mechanistically, an M1 agonist induces the same effects as ACh via M1 mAChR; and c) some muscarinic agonists have shown positive cognitive effects similar to those obtained with AChE-Is (reviewed in refs. 4 and 8). It is also unlikely that the beneficial cognitive effects of these AChE-Is are mediated entirely by receptors other than the mAChRs (i.e., only nicotinic receptors). Notably, several nicotinic agonists are developed for AD treatment. However, nicotinic receptors are decreased in AD brains (3), hampering perhaps the effectiveness of this therapeutic strategy in AD. On the other hand, one cannot ignore the possibility that better effects might be obtained with a combined treatment that stimulates both post-synaptic mAChR (e.g., M1 mAChR) and brain nicotinic receptors.

5.1. Some muscarinic drugs had major clinical limitations

Most studies with muscarinic agonists employed clinical protocols designed to show their use in a symptomatic treatment on cognition and behavior in AD patients. Unfortunately when studies failed, in particular Phase III, these were performed with compounds having several major clinical limitations (reviewed in ref. 8). The sponsors (drug companies) without a clear scientific reasoning prematurely interrupted several clinical studies on other muscarinic agonists. All these limitations did not allow a proper evaluation of the clinical concept. Furthermore, very few of the already developed agonists could fulfill rigorous acceptance criteria (8) in order to be considered as a promising treatment strategy in AD patients.

1. In general, there is a dearth of centrally active M1 agonists (4, 6 – 9). The published functionally selective M1-selective agonists are partial agonists, at least in some of the standard assays. Compounds like milameline and sabcomeline, lack selectivity for M1 mAChR and thus cannot be termed as M1 agonists (4, 8, 15 – 17). Some of the tested agonists like alvameline (18) are very weak agonists for M1 mAChR. In fact, these can be considered more as M1 antagonists (8). Notably, M1 antagonists can be detrimental on cognitive functions.

2. Some of the clinical studies employed drugs that had extremely low oral bioavailability and extensive metabolism [e.g., xanomeline, M4>M1 agonist, has a bioavailability in humans of <1% (13, 14)], leading to adverse effects (13) mediated most probably by a plethora of receptors (8). Notably, AChE-Is that are effective in AD have an incomparable better bioavailability (%): donepezil (100%), metrifonate (90%), galanthamine (100%), rivastigmine (36%) and tacrine (17%) (55).

3. Most of the drugs that failed in AD had a narrow safety margin with several side effects that limit the number of patients to those who can tolerate higher and perhaps more effective doses. These include agonists such as milameline, xanomeline, sabcomeline and alvameline (reviewed in ref. 8). A compound with a minimal safety margin of >100 would be preferred. However, sabcomeline, for example, shows only 3 – 10-fold separation of cognitive vs side effects (8). This extremely limits the dosing protocol in AD patients. Milameline produced a plethora of side effects including such toxic effects as corneal opacities and urinary tract sepsis in preclinical studies and gastrointestinal, flatulence and parkinsonian symptoms in clinical trials (reviewed in ref. 8).

4. All these muscarinic drugs were administered in fixed non-individualized doses, that for the non-selective agonists produced intolerable side effects.

5.2. Phasic versus tonic stimulation: what is really needed in AD treatment?

ACh released pre-synaptically when required causes phasic stimulation. Released ACh activates post-synaptically M1 and M3 mAChR, while M2 mAChR autoreceptors (presynaptic) control via negative feedback the concentration of this neurotranmitter in the synapse. The entire cholinergic signal in a normal synapse is extremely well controlled both pre- as well as post-synaptically. Tonic stimulation is induced by post-synaptic activation of mAChR either by constant high concentration of ACh or an exogenously administered muscarinic agonist. Whether cholinergic treatment in AD should produce its beneficial effect on cognition via a phasic or tonic stimulation is less than clear as outlined below:

1. The assumption is that AChE-Is should be more effective in intact synapses in AD and less in those synapses where there is already a presynaptic cholinergic hypofunction. AChE-Is increase the efficiency of cholinergic transmission by preventing the hydrolysis of released ACh, thus making more ACh available at the cholinergic synapse. This may be conducive to an over-excitation of intact synapses, perhaps in a tonic rather than phasic manner. Like muscarinic agonists, AChE-Is are assumed to take advantage of the relative preservation of post-synaptic mAChR in AD (56). In this context, the longer the inhibition of AChE, the better is the effect of the drug (e.g., donepezil or metrifonate vs physostigmine) on cognition in AD patients (55). In case of prolonged inhibition of AChE by an AChE-I, the stimulation produced by elevated levels of ACh in the cholinergic synapse cannot be considered as phasic. The synaptic concentration of elevated ACh may well exceed the normal level needed to activate post-synap-

tic mAChR (M1, M3). In such a scenario, there is prolonged activation of these receptors. In fact, these receptors may be "tonically" activated (the synapse may even be "flooded" by ACh), (see also ref. 57). Thus it is not conceivable to presume that the beneficial effects of AChE-Is in AD patients is due to a phasic activation.

2. Microdialysis studies in rat brains with the FDA-approved AChE-Is show threefold or greater increases in ACh in the extracellular fluid in hippocampus in rats (58, 59). The extracellular ACh concentration measured by the intracerebral microdialysis technique reflects the ACh concentration in the synaptic cleft (59). Thus the synaptic level of ACh must be high if ACh level is increased to that extent and it is hard to imagine this producing a phasic response. In fact, AChE-Is may work tonically just like a post-synaptic agonist.

3. The apparent phasic aspect of AChE-I relates to the presynaptic inhibition of further release of ACh. This, in fact, may be considered a drawback for using AChE-Is in AD. Notably, excessive autoreceptor stimulation (e.g., M2 mAChR) may eventually reduce the ability of pre-synaptic neurons to transmit properly. Thus the activation of presynaptic autoreceptors may play a role in reducing the efficacy of AChE-Is. Moreover in AD, AChE needs to be inhibited to such a degree that the released ACh can function post-synaptically without affecting the pre-synaptic events necessary for renewed transmitter synthesis and release (60). This excludes a clinical response that is phasic and in fact emphasizes the tonic aspect.

4. Beneficial effects on cognition in AD patients can be detected with the standard AD Assessment Scale-cognitive (ADAS-cog) test after a few weeks of chronic treatment with either an AChE-I or a muscarinic agonist. Neither a phasic nor a tonic stimulation can provide an intelligent explanation for such a delayed effect. Xanomeline improved cognitive deficits in AD patients comparable to the effects observed with AChE-Is (13). However, behavioral effects of AChE-Is in AD patients may differ when compared with the beneficial pronounced effects of xanomeline on this clinical parameter. The pharmacokinetic profile in the case of a muscarinic agonist may not reflect accurately pharmacodynamic effects relating to cognitive and psychotic behaviors in AD patients. Notably, the maximal effects of xanomeline on cognition in AD patients occurred after 12 weeks, whereas the behavioral improvements were observed much earlier (13). In such a scenario, there is no practical relevance to tonic or phasic activation of the receptors.

5. How can a phasic stimulation be induced? One feasible approach is to administer either choline [or phosphatidyl choline (lecithin)], the physiologic precursor of ACh biosynthesis. This approach can increase, in principle, ACh levels in brain of animals and in plasma and CSF of humans, but this strategy failed in AD as it did not improve cognition in AD. Although various arguments can be proposed to explain ths lack of clinical efficacy, it is evident that a phasic stimulation alone may not be sufficient for a cholinergic replacement treatment to be effective in AD.

6. Studies in several animal models, in particular those modeling the cholinergic deficiency in AD using either AChE-Is or muscarinic agonists, do not indicate a major role of phasic vs tonic modulation in ameliorating cognitive impairments. Notably, when selective partial M1 agonists were compared with the FDA-approved AChE-Is (tacrine, donepezil, and rivastigmine), these agonists were at least as effective as these AChE-Is in restoration of cognitive impairments (4, 8).

7. ACh released (from genetically modified cells that produce ACh) into denervated neocortical target regions is sufficient for improving cognitive function in rats. The released ACh presumably acts in a tonic manner on post-synaptic receptors, as postulated for dopamine released from grafted non-neural cells (61).

8. Preclinical studies indicate that memory and learning are slow processes. Activation of mAChR can induce expression of immediate early genes and transcription factors that give rise to the delayed expression of functional and morphological changes that underlie neurotrophic activity, learning and memory and other processes (62). As a result of such mechanisms, cognitive effects induced by AF102B (63), an M1 agonist (tonic?), or by BINB99 (64), an M2 antagonist (phasic?), have a long duration of action which lasts long after the compound is no longer detectable in the body fluids or brain regions (63, 64). Since M1 muscarinic agonists might also have neurotrophic-like effects, a compound with a moderate half-life can induce eventually long term effects reminiscent of endogenous neurotrophins (4, 8). In such a scenario, the relevance of tonic vs phasic activation of the mAChR is questionable.

9. An argument that was raised against the use of muscarinic agonists in general was that such compounds via a tonic stimulation might produce tolerance following a long term-treatment. However, no strong evidence supports such a claim since most of the drugs tested in clinical trials were partial agonists, that unlike full agonists, are not supposed to induce tolerance.

In summary, the disappointing results obtained so far in AD patients with some of the tested muscarinic agonists compared with AChE-Is cannot be attributed to a tonic response associated with agonist stimulation.

5.3. Changes in signal transductions in AD and vicious cycles

The number of M1 mAChR is relatively preserved in AD, but there appears to be impairment in receptor G-protein interaction (reviewed in refs. 2, 3, 57 and 65). This

impairment may occur at several loci (57). The cholinergic hypofunction in AD can be linked with formation of Aβ (2, 4, 8, 21, 23, 65). Furthermore, coupling of M1 mAChR with G-proteins in the hippocampus is impaired following a septal-hippocampal cholinergic lesion (66) and in hippocampal areas most affected by Aβ plaques, but is intact in less affected areas (2, 3). Notably, subtoxic levels of Aβ disrupted mAChR coupling to G-proteins linked to phospholipid hydrolysis without producing neuronal cell injury or death (67). The mechanism underlying Aβ-induced disruption of mAChR-G-proteins coupling is not completely understood, yet it presumes involvement of reactive oxygen species (ROS) since this uncoupling can be attenuated by antioxidants (67). This impairment in coupling can lead to decreased signal transduction, to a reduction in levels of trophic α-APPs and generation of more Aβ (3, 4, 8, 65, 66). Aβ at very low concentrations can also suppress ACh synthesis and release, aggravating further the cholinergic deficiency (68, 69). Some of these "vicious cycles" (Fig. 2B) may be blocked by M1-selective agonists (4, 8).

Another pertinent finding relates to altered signal transduction in AD in the cAMP-signaling pathways. Notably, cAMP levels were found to be increased in CSF taken from AD patients. The elevated levels of CSF cAMP in AD patients correlated significantly with CSF *tau* protein levels, indicating an up-regulation of cAMP-signaling pathway in AD physiopathology (70). Interestingly, mRNA for the α-subunit of Gs (the G-protein known to activate adenylate cyclase) were markedly elevated in postmortem brain tissues of AD brains (71). In this context, we have proposed that the desired M1selective agonists for the treatment of AD, should not stimulate adenylyl cyclase via M1 mAChR, but should still be able to activate the Gq/11 (4, 6, 20). Some M1 agonists including those from the AF series may fulfill these requirements.

What might be some of the potential consequences of increased cAMP, in general, and due to mAChR activation, in particular?

In general, intracellular elevation of cAMP may result in inhibition of both constitutive and PKC-stimulated secretory cleavage of APP (72). In particular, less stimulation of reduced M2 mAChR in AD by ACh would reduce Gi activation, the G-protein that mediates inhibition of adenylate cyclase. This could lead to elevated cAMP levels, which could activate cAMP-dependent protein kinase (protein kinase A). Protein kinase A, which about one third of it is associated with microtubule-associated proteins, can overphosphorylate *tau* proteins (see review, ref. 65). It is possible that a combined loss of ACh-induced presynaptic signaling, together with post-synaptic activation of M1 (and M3 mAChR)-mediated elevation in cAMP [by a very potent agonist (e.g., a full M1 agonist) or increase in ACh levels (due to inhibition of AChE)] contribute to activated kinases which progressively can elevate hyperphorylated *tau* (see reviews, refs. 2, 4 and 65). Notably, hyperphosphorylated *tau* is the primary component of

Table 2. The relation between cholinergic function (muscarinic, nicotinic or both) and *tau* hyperphosphorylation

Experiment (Ref.)	Summary
PC12M1 cell cultures +/− AF102B, carbachol (44)	Stimulation of M1 mAChR *decreases tau* phosphorylation and *increases tau* dephosphorylation.
CHO-M1AChR co-transfected with *tau* and GSK-3β +/− carbachol, xanomeline, sabcomeline (45)	Muscarinic agonists *decrease tau* phosphorylation through PKC activation and via GSK-3β inhibition. Carbachol induces a *decrease* in *tau* phosphorylation and an *increase* in the formation of microtubule bundles.
Primary cortical cell cultures +/− carbachol (45, 46)	Carbachol induces a *decrease* in *tau* phosphorylation.
ApoE-deficient mice versus Control (47)	*Tau* proteins in ApoE-deficient mice contain a hyperphosphorylated "hot spot" domain, localized N-terminally to the microtubule binding domain.
ApoE-deficient mice and Control +/+ AF150(S) (0.5 mg/kg, p.o., 5 days/week for three weeks), respectively (47)	*Tau* epitopes which reside in the hyperphosphorylated "hot spot" are *dephosphorylated* by AF150(S), but unaffected in controls. *Tau* epitopes which flank the "hot spot" domain are *dephosphorylated* by AF150(S) in both groups. Epitopes located at the N- and C-terminal of *tau* are *unaffected* by AF150(S) in both groups.
Other cholinergic compounds (23)	
Nicotinic agonists (nicotine, epibatidine) AChE-Is (tacrine, donepezil, galanthamine) [SH-SY5Y cells]	Induce an increase in both phosphorylation and dephosphosphorylation of *tau*

paired helical filaments that form neurofibrillary tangles in AD brains (43). If this hypothesis is valid, it may indicate that M1 agonists that do not activate adenylate cyclase should be preferred in treatment of AD. Furthermore, it may indicate a detrimental effect of some highly efficacious or very potent muscarinic agonists that can activate all M1 mAChR-mediated signal transductions in a promiscuous way including the M1 mAChR coupling with Gs. The same caution may apply to AChE-Is since in such a scenario, elevated ACh levels can again increase brain cAMP levels.

The above scenario may be complementary to the opposing effects of M1 agonists vs nicotinic agonists and AChE-Is (23) on *tau* phosphorylated and dephosphorylated levels (Table 2). It is not yet clear whether the increased phosphorylation of *tau* proteins induced by nicotinic agonists and AChE-Is, in vitro, may be of any relevance to AD (a long-term detrimental effect?). Nevertheless, a decreased phosphorylation of *tau* protein via M1 mAChR activation may indicate a linkage between the muscarinic signal transduction system(s) and the neuronal cytoskeleton, via regulation of phosphorylation of *tau* microtubule-associated protein (2, 44–47). It can be speculated that activation of M1 mAChRs might provide a novel treatment strategy for AD by modifying *tau* processing in the brain.

6. Conclusions and future perspectives

The text above and other recent publications (2, 4, 6–9, 23, 57) indicate that a therapeutic strategy in AD patients based on M1 muscarinic agonists is probably not wrong, yet it requires a critical reassessment. This can be achieved mainly by a modification of our current thinking concerning this approach combined with additional research. Failure of some muscarinic agonists that had *a priori* major clinical limitations cannot be used against this therapeutic concept. Definitively it should not discourage the clinical development of better M1 agonists for treatment of AD patients. History teaches us that sometimes the first-generation therapeutics, even when they failed, paved the way to more effective second-generation drugs with a similar mechanism, providing there is a sensible hypothesis that can support such an approach. In this context recent scientific findings indicate that the basic approach is still valid, yet the original cholinergic hypothesis regarding M1 agonists was oversimplistic. Furthermore, these novel findings strengthen an updated and modified hypothesis that the M1 muscarinic treatment strategy is a viable and rational approach that merits to be advanced into AD patients and other populations at risk to develop AD (e.g., minimal cognitive impairment). Proof of the clinical concept, using highly selective M1 agonists free of the disadvantages of the earlier agonists, will require a strong multidisciplinary collaboration between preclinical and clinical researchers and of course pharmaceutical companies.

M1 mAChR activation, *inter alia*, induces neurotrophic-like responses (73–76), promotes normal processing of APP and decreases *tau* phosphorylation. It appears that activation of M1 mAChR could beneficially modulate dysfunctions that are associated with AD including – Aβ and *tau* proteins, ApoE, as well as some processes involving certain G-proteins and neurotrophins (reviewed in refs. 4 and 8). The compound with a high safety profile and selectivity for M1 mAChR, in particular in vivo, should be selected as a therapeutic strategy designed to influence the progression of AD and the onset or prevalence of various target populations at risk to develop AD. Based on current available data, select compounds from the AF series and some other highly selective M1 agonists may well fulfil such rigorous acceptance criteria.

Several therapeutic strategies in AD are extensively pursued, most focused on modulation of APP metabolism and secretion, Aβ formation and/or aggregation (20). However, Aβ is a natural constituent of body fluids. Therefore it is not known how inhibition of its formation would affect normal function. Inhibition of β- and/or γ-secretases would lead to accumulation of APP or its Aβ-containing fragments, if the inactivated α-secretase remains unmodified. These fragments can be potentially neurotoxic. Therefore, attempts to reduce Aβ in AD should also target the α-secretase. In this context, the ability of M1 agonists to stimulate APPs secretion and Aβ reduction (via activation of α-secretase) might lead to a relatively direct route of Aβ reduction. This was already evidenced in AD patients. Thus two clinical studies revealed that chronic treatment with M1 agonists (AF102B and talsaclidine) reduced significantly cerebrospinal fluid (CSF) Aβ (total) in AD patients (77, 78). These pioneering studies may indicate that M1 agonists have an important role in affecting Aβ processing, probably by reducing Aβ burden in AD patients. No other compounds were yet reported with such a unique profile. Moreover, physostigmine (an AChE-I) and hydroxycholoroquine (an anti-inflammatory drug) did not show a significant effect on CSF Aβ levels when tested in AD patients in the same study of AF102B (78). Additionally, in other studies, two of the AChE-Is effective on cognition in AD [donepezil (aricept) and galanthamine] did not show any significant effects on CSF Aβ levels in AD patients (79). All these may emphasize the unique therapeutic value of M1 agonists vs the oversimplified concept that such compounds should provide mainly a symptomatic treatment.

It is not currently known whether cholinergic degeneration, amyloid plaques, or neurofibrillary tangles is the most important or the major lesion in AD. Also how these new findings may affect the course of the disease or influence the onset of AD are questions that remain to be answered.

However the cholinergic system appears to participate in the processing of APP and *tau* phosphorylation (see review 23).

Albeit initially designed to act as a symptomatic treatment in AD, M1-selective muscarinic agonists may have the potential to impact also on different aspects of AD pathogenesis regardless which is the main culprit in its induction (e.g., Aβ, *tau* hyperphosphorylation, ACh deficiency or their various combinations) (this review and refs. 6 and 8). M1-selective agonists may allow testing effects on Aβ and *tau* in AD patients, perhaps more directly than with other tools. This may modify eventually the classical cholinergic hypothesis in AD that was limited to a symptomatic treatment and ignored the added value of M1 agonists as potential disease-modifying agents. Chronic studies with highly selective M1 agonists in animal models mimicking various aspects of the AD pathology (e.g., transgenic animals which express one or several pathogenic proteins) followed by clinical studies (e.g., early stage of AD patients and/or other populations at risk), are needed to test this unifying hypothesis.

A muscarinic treatment of AD not only will modify our current thinking about such treatments, but it may enlarge their use in combination therapy and in other neurological, neuropsychiatric diseases and autoimmune diseases with a documented cholinergic deficiency. In this context, it is relevant to mention the recent FDA approval of EVOXAC™ (Cevimeline, AF102B) for treatment of dry mouth in Sjogren Syndrome granted to Snow Brand Pharmaceuticals, Inc. (USA) [subsidiary of Snow Brand, Japan, the licensee of this compound]. This drug might prove to be effective in the future in other aging-related diseases such as AD.

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